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A CONSERVED CYSTEINE CLUSTER, ESSENTIAL FOR TRANSCRIPTIONAL ACTIVITY, MEDIATES HOMODIMERIZATION OF HUMAN METAL-RESPONSIVE TRANSCRIPTION FACTOR-1 (MTF-1)

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Abstract

Metal-responsive transcription factor-1 (MTF-1) is a zinc finger protein that activates transcription in response to heavy metals such as Zn(II), Cd(II) and Cu(I) and is also involved in the response to hypoxia and oxidative stress. MTF-1 recognizes a specific DNA sequence motif termed the metal response element (MRE), located in the promoter/enhancer region of its target genes. The functional domains of MTF-1 include, besides the DNA-binding and activation domains and signals for subcellular localization (NLS and NES), a cysteine cluster ^{632}CQCQCAC^{638} located near the C-terminus. Here we show that this cysteine cluster mediates homodimerization of human MTF-1, and that dimer formation *in vivo* is important for basal and especially metal-induced transcriptional activity. Neither nuclear translocation nor DNA binding are impaired in a mutant protein in which these cysteines are replaced by alanines. Although zinc supplementation induces MTF-1 dependent transcription it does not per se enhance dimerization, implying that actual zinc sensing is mediated by another domain. By contrast copper, which on its own activates MTF-1 only weakly in the cell lines tested, stabilizes the dimer by inducing intermolecular disulfide bond formation and synergizes with zinc to boost MTF-1 dependent transcription.

Key words: MTF-1; heavy metal homeostasis; gene expression; homodimerization; cysteine cluster.

1 Introduction

A typical transcription factor is composed of several functional domains that regulate its DNA binding, transcriptional activity and subcellular distribution. Metal-responsive transcription factor-1 (MTF-1), which is conserved from mammals to insects, is activated by heavy metals [1-3] and other stressors, like hypoxia [4] and oxidative stress [5]. Despite extensive research the protein domains that regulate its activity in response to these stresses are still not all identified.

MTF-1's DNA-binding domain consists of six zinc fingers that mediate binding to the cognate DNA motif termed metal-response element (MRE), with a core consensus sequence "TGCRCNC". MREs are often found in multiple copies in the promoter/enhancer region of MTF-1 target genes, of which the metallothionein genes are the best studied ones. Other target genes include *ZnT-1*, encoding a zinc efflux transporter [6], and the cadmium-responsive genes selenoprotein W, muscle 1 gene (*Sepw1*), N-myc downstream regulated gene 1 (*Ndrg1*) and cysteine- and glycine-rich protein 1 gene (*Csrp1*) [7]. Taking the fact that MTF-1 requires elevated levels of zinc in cell free DNA binding studies [3] together with the observation that the different zinc fingers have different zinc-binding affinities [8,9] implies that at least some of its zinc fingers participate in sensing cellular zinc levels. Studies on the role of individual zinc fingers in zinc sensing have yielded ambiguous results so far. There is evidence that zinc fingers 1 to 4 represent the core DNA-binding domain and that zinc fingers 5 and 6 can act as zinc sensors to mediate metal-responsive transcription [10]. Other studies imply that zinc finger 1 and the "linker" peptide between fingers 1 and 2 are involved in zinc sensing [11,12].

Based on their characteristic amino acid compositions [13], three transcriptional activation domains were ascribed to MTF-1 [14], each of them showing transcriptional activity when

fused to a heterologous DNA-binding domain. The acidic activation domain confers the strongest activity and is also able to mediate metal-induced transcription when fused to the DNA-binding domain of the yeast transcription factor Gal4, at least in a subset of the cell lines that were tested [15]. Additionally, kinase inhibitor studies implied that MTF-1 is regulated, directly or indirectly, by phosphorylation through the action of protein kinase C, tyrosine kinases, casein kinase II and c-Jun N-terminal kinase [16,17].

Under standard conditions, MTF-1 mainly resides in the cytoplasm but rapidly translocates to the nucleus upon metal load [18,19]. Subcellular localization is regulated by a non-conventional nuclear localization signal (NLS), which spans the first three zinc fingers. In addition, a cluster of basic amino acids located N-terminal to the zinc finger region contributes to nuclear import as an “auxiliary” NLS [15,18]. A nuclear export signal (NES) overlapping with the acidic activation domain confers Leptomycin B-sensitive/Crm1-dependent nuclear export to MTF-1. The functional relevance of export is unclear, as a constitutively nuclear protein is still able to mediate metal-responsive transcription [15].

A cluster of four cysteines ^{632}CQCQCAC^{638} close to the C-terminus of human MTF-1 is conserved in all vertebrate orthologs (Figure 1A and B). It was previously shown that single and double substitutions of the cysteines in the cluster decrease the metal-induced transcriptional response, whereas nuclear accumulation and DNA binding upon metal stress are unaffected [20,21]. A domain with this particular spacing of cysteines was thus far not described for other transcription factors and the mechanism of its function remained unclear.

Interestingly, other metal-responsive transcription factors in lower eukaryotes contain regulatory cysteine clusters with different overall amino acid sequences that serve diverse functions. Cuf1 of the fission yeast *S. pombe* activates expression of genes for copper import under copper deprivation [22]. This transcription factor contains a cysteine-rich motif within

its C-terminal region that, when bound by copper, blocks the nuclear localization signal via an intramolecular interaction and thereby prevents target gene transcription [23]. In *S. cerevisiae* the metal-sensitive transcription factor Mac1 also responds to low copper availability [24,25] but via a different mechanism. Its activation domain harbors two cysteine-rich regions, REP-I and REP-II. In the copper-bound state REP-I induces an intramolecular interaction between the transactivation domain and the DNA-binding domain, thereby inhibiting both functions [26]. Another transcription factor of baker's yeast, Ace1, activates transcription of the *CUP1* metallothionein gene in response to copper load [27]. Here, copper binds to a cluster of several cysteines overlapping the DNA-binding domain and induces a conformational change that allows DNA binding [28,29].

In the present study, we show that the cysteine cluster of human MTF-1 mediates homodimerization and that metal-induced transcription depends on this dimerization. Nucleo-cytoplasmic shuttling and DNA binding are not affected in the cysteine cluster mutant that is unable to dimerize. Exposure to elevated concentrations of zinc, a condition that induces the transcriptional activity of MTF-1, did not increase dimerization, demonstrating that even though dimerization via the cysteines is a prerequisite for metal-induced transcription, it is not participating in the process of zinc-sensing. However copper, which on its own activates human MTF-1 poorly in the cell lines tested, stabilizes the dimer through oxidation of cysteines and synergizes with zinc to boost transcription.

2 Materials and methods

2.1 Cell culture and transfections. HEK293, HEK293T (human embryonic kidney), U2OS (human osteosarcoma), HeLa (human cervix carcinoma) and mouse MTF-1-/- fibroblast-type

115 cells (dko7 [3,14]), were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco)
116 containing 8 % fetal bovine serum (Biochrom AG), 100 U/ml penicillin, 100 µg/ml
117 streptomycin and 2 mM L-glutamine (Invitrogen). For transfections with the calcium-
118 phosphate method, herring sperm DNA was added to each sample to a total amount of 20 µg
119 DNA per 10 cm dish. Cells were washed 14 to 16 hrs after transfection. Unless mentioned
120 otherwise, metal treatments were done 40 hrs after transfection for four hrs by
121 supplementing the medium with metal salt to the indicated concentration.
122
123 *2.2 Plasmid constructions.* The expression vector containing a VSV-tagged human MTF-
124 1 cDNA clone (aa1-743) under the control of the CMV promoter (hMTF-1-VSV) was
125 described previously [18]. The expression vector in which cysteines at positions 632, 634,
126 636 and 638 were replaced by alanines (CYSmut-VSV) and the vectors containing single
127 substitutions were generated based on hMTF-1-VSV using site directed mutagenesis
128 (QuikChange, Stratagene) according to the manufacturer´s instructions. The expression
129 plasmids containing FLAG-tagged MTF-1 truncations (FLAG-1-321, FLAG-1-511) were
130 produced by cloning corresponding PCR fragments into the EcoRV/XhoI sites of pCATCH-
131 FLAG [30]. FLAG-322-end, 322-endCYSmut-VSV, 322-end-VSV, FLAG-hMTF-1 and FLAG-
132 CYSmut were generated using PCR technology and restriction enzyme digestion/ligation
133 based on the described plasmids. The reference and reporter plasmids CMV-OVEC-Ref,
134 OVEC-Ref (SV40 promoter) [31], 5xGOVEC (containing five Gal4 binding sites in its
135 promoter region) [32], 4xMREd-OVEC [31] were described previously. 2xGOVEC, 4xGOVEC
136 and 8xMREd-OVEC were produced by fusing corresponding annealed oligonucleotides into
137 the OVEC-vector [31]. Gal4 DNA-binding domain fusion constructs are based on the vector
138 pSCTGal(1-93) containing the DNA-binding domain (amino acids 1-93) of the yeast

transcription factor Gal4 [13]. To generate VP16-fusion constructs the acidic transcriptional activation domain of the viral VP16 protein (amino acids 413-490 [13]) was fused to various human MTF-1 clones. Sequences of the oligonucleotides used and detailed cloning strategies are available on request. The p300-FLAG expression plasmid was a generous gift of Dr. Michael Hottiger (Institute of Veterinary Biochemistry and Molecular Biology, University of Zürich).

2.3 Co-immunoprecipitations and immunoblotting. Cell lysates, from a confluent 10 cm dish of HEK293 or HEK293T cells transfected with 2 µg of each expression plasmid, were prepared in a lysis buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5 % NP-40, 1 mM DTT and protease inhibitors. The cleared lysates were incubated with 1 µl anti-VSV antibody (Sigma, V5507) for 1 h at 4 °C, followed by 1 h of incubation with 20 µl of Protein-G Sepharose Fast Flow (GE Healthcare). The Sepharose beads were washed four times with lysis buffer and precipitates were eluted by boiling 5 minutes in Laemmli buffer containing 2.5 % β-mercaptoethanol. Proteins were separated by 7.5 or 10 % tris-glycine SDS-PAGE (BIO-RAD) and transferred on a PVDF-membrane (Amersham). VSV- and FLAG-tagged proteins were detected using 1:10'000 and 1:1'000 dilutions of anti-VSV (Sigma, V5507) and anti-FLAG (Sigma, F1804) antibody, respectively, followed by a horseradish peroxidase-conjugated anti-mouse IgG at a 1:10'000 dilution (GE Healthcare, NA931). Proteins were visualized using the ECL chemiluminescent detection system (Pierce).

2.4 S1 nuclease protection assay. Exponentially growing cells of a 10 cm culture dish were transfected with 10 µg of the indicated reporter plasmid and 1-5 µg of reference plasmid (1 µg CMV-OVEC-Ref for U2OS cells, 5 or 3 µg OVEC-Ref for dko7 or HeLa cells,

163 respectively). The amounts of transfected expression clones per 10 cm dish are the
164 following: 2 µg of wild type or mutant MTF-1 expression vectors in Figure 6; 2 µg of Gal4-
165 and VP16-fusion constructs in Figure 4 and 5a; 0.05 µg of Gal4-fusion clone in Figure 6.
166 Isolation of RNA and the S1 nuclease protection assay were done as described previously
167 [31,33]. Signals were visualized using the fluorescent image analyzer FLA-7000 and
168 quantified using the ImageGauge software (Fujifilm life science). Reporter signals were
169 normalized to the reference signals.
170
171 *2.5 Preparation of nuclear extracts and electric mobility shift assay (EMSA).* Preparation
172 of nuclear extracts from transiently transfected HEK293T cells was performed according to
173 [34]. Binding reactions and gel conditions were described in [2]. For Figure 3C 30 µg of
174 nuclear extract and 600 pmol of end-labeled MRE-s oligonucleotide was used.
175
176 *2.6 Chemical crosslinking.* Cell lysates were prepared using a buffer containing 20 mM
177 HEPES pH 8, 150 mM NaCl, 1 % Triton X-100, 5 mM DTT and protease inhibitors. Protein
178 concentrations were measured with the Bio-Rad protein assay (Bio-Rad); 3.7 µg/µl of protein
179 were incubated with 5 mM EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide
180 hydrochloride) for 30 minutes at room temperature in the dark. The crosslinking reaction was
181 stopped by adding Laemmli sample buffer containing 2.5 % β-mercaptoethanol and samples
182 were analyzed using SDS-PAGE.
183
184 **3 RESULTS**
185

3.1 Mutation of the four cysteines of the cluster hampers transcriptional activity. A MTF-1 mutant in which the cysteines at positions 632 and 634 were replaced by alanines was previously shown to be strongly reduced in transcriptional activity [20]. In the mutant protein used in the present study all four cysteines of the cluster were replaced by alanines, hereafter referred to as cysteine mutant ("CYSmut" in figure legends). The transcriptional activity was analyzed in a mouse cell line lacking MTF-1 (dko7) by using MRE-containing reporter constructs. Wild type MTF-1 induced expression of a reporter containing four tandem MREs (4xMREd-OVEC) 6- and 8-fold in response to 100 µM zinc and 50 µM cadmium, respectively (Supplementary Figure 1A). Mutation of all four cysteines almost completely abolished zinc- and cadmium-induced transcription, which is consistent with previous results obtained by Chen and coworkers using the C632/634A mutant [20]. Even the basal activity was reduced to 50 % of wild type level, indicating that the cysteines, besides their major role in mediating strong metal-induced transcription, also contribute to basal transcriptional activity. The cysteine mutant also failed to drive transcription from authentic promoter segments of mouse *MT1* (metallothionein 1) and human *MT2a* (metallothionein 2a) (Supplementary Figure 1B).

We also investigated the cytoplasmic-nuclear translocation for the wild type and the cysteine mutant by indirect immunofluorescence in human U2OS and HEK293T cells. In line with the results obtained for the C632/634A double mutant [20] we observe that the cysteine mutant is still able to translocate to the nucleus (Supplementary Figure 2A and 2B). Likewise we also find that the cysteine mutant is still able to bind to the MRE in a bandshift assay (Supplementary Figure 2C). Altogether these data show that the inability of the mutant protein to activate transcription is not due to a failure in DNA binding or nuclear translocation.

3.2 MTF-1 homodimer formation depends on the cysteine cluster. In analogy to the studies with the yeast transcription factors Mac1 and Cuf1, we tested whether the cysteine cluster of MTF-1 makes an essential intramolecular interaction with other regulatory regions, such as the zinc fingers, the NLS and NES sequences and/or the acidic- and proline-rich activation domain. We performed co-immunoprecipitation with lysates of transiently transfected cells with differentially tagged versions of MTF-1 subsegments. No interaction could be detected, even if the subsegments overlapped, between the N-terminal (aa1-511 or aa1-321) and the C-terminal domain (aa322-end) (Figure 2A). However, the full length proteins interacted (Figure 2B). The C-terminal half, missing the zinc finger region, was sufficient to mediate dimerization (Figure 2C), which indicates that the interaction is inter-, rather than intramolecular, and that it can occur independently of DNA binding.

We next tested, by means of co-immunoprecipitation, if mutation of the cysteines abolishes the observed dimerization. As shown in Figure 3A the interaction is almost completely abrogated. The fact that interaction was equally lost if one or both proteins are mutated in this assay is compatible with a model in which dimerization occurs between the cysteine clusters of both proteins, rather than between a cysteine cluster on one partner and a different domain in the other. To determine the stoichiometry of the MTF-1 complex we performed a crosslinking reaction with the zero length cross-linker EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), which produces isopeptide bonds between carboxyl groups and primary amines. Cell lysates of HEK293T cells transiently transfected with the C-terminal construct (aa322-end) were treated with 5 mM EDC for 30 minutes. Comparison with the molecular weight standards revealed that the slower migrating band in EDC treated cell lysates corresponds to a dimer (Figure 3B). In accordance with the immunoprecipitation results, the dimer band was not observed for the cysteine mutant

protein. The amount of crosslinked dimer in our assay was relatively low, presumably because the optimal pH for crosslinking with EDC is at a non-physiological pH between 4.7 and 6.0, while the crosslinking reaction showed here was carried out at pH 7.9 becaus no crosslinking of MTF-1 and no co-immunoprecipitation of MTF-1 dimers was observed at pH 7.0 or lower (not shown). Therefore we cannot determine from these data whether only part or all of MTF-1 is present in the dimeric form *in vivo.*

In a standard bandshift assay there was no evidence for MTF-1 dimerization resulting in a supershift, presumably because the conditions during gel electrophoresis were unfavorable to maintain the dimer. However, by raising the concentration of both MTF-1 and labeled probe in the binding reaction a slower migrating band was formed (Figure 3C). If the C-terminal half was coexpressed with the full length protein, an intermediate size band was observed, indicating a heterodimer formed between a full length MTF-1 and the C-terminal fragment. This intermediate band was absent if the four cysteines of the C-terminal cluster were mutated, consistent with the finding that the protein-protein interaction is dependent on the cysteine cluster.

To see if a metal ion, especially zinc, is part of the dimerization reaction, we performed a co-immunoprecipitation in the presence of the metal chelators 1,10-orthophenanthroline or TPEN, which show a high affinity to zinc and other divalent metals. This experiment was done with the C-terminal construct, since adding chelators might remove zinc from the N-terminal zinc fingers and lead to misfolding and aggregation of the proteins. None of the chelators eliminated the dimerization reaction of the C-terminal MTF-1 construct (Figure 3D), which might mean that dimerization does not involve divalent metal ion coordination, or that metal ions are tightly packed in-between the cysteines and thus inaccessible to the chelators.

3.3 MTF-1 homodimerization is not increased by zinc treatment. To assess if the extent of dimerization is responsive to metal treatment and thus might be an important component of metal sensing by MTF-1, we also pretreated the cells for four hrs with zinc prior to cell lysis and co-immunoprecipitation. This did not change the apparent strength of the interaction, even if additional zinc was added to the lysis buffer (data not shown).

A similar result was obtained with an independent approach, a mammalian two-hybrid assay performed in human U2OS cells. A C-terminal segment (aa 515-end) of MTF-1 was fused either to the DNA-binding domain of the yeast transcription factor Gal4 (aa1-93) or to the activation domain of the herpes simplex virus transcription factor VP16. Of MTF-1's three activation domains only the serine/threonine-rich on is present in these constructs, and the resulting Gal4 fusion protein showed weak transcriptional activity on its own (see below, Figure 5A). When the two constructs, Gal4-515-end-VSV and 515-end-VP16, were coexpressed we observed robust reporter gene expression. The interaction and the resultant reporter gene expression were severely reduced by mutation of the four cysteines in one or both partners (Figure 4). Single substitutions had a lesser effect, resulting in only a slightly reduced two-hybrid signal (data not shown). Pretreatment of the cells with zinc for four hrs, a treatment which increases MTF-1's transcriptional activity, did not boost the transactivation of the reporter gene, supporting the notion that the cysteines are not used for zinc sensing (Figure 5A). Even more prolonged treatment with zinc had no effect on the intensity of the two-hybrid interaction.

3.4 Copper pretreatment induces dimerization via formation of intermolecular disulfide bonds and synergizes with zinc for transcription. As mentioned before, zinc treatment did not enhance the interaction between the cysteine clusters. Cadmium and copper were tested in

a time course experiment to investigate if these metals increase the homodimerization of MTF-1. Both copper and cadmium boosted the two-hybrid interaction (Figure 5A). We also tested menadione, a drug that, like copper, undergoes redox cycling and induces oxidative stress. However, menadione failed to induce the dimerization, indicating that this effect is restricted to metals like copper and cadmium, which can cause oxidative stress. When lysates of HEK293T cells overexpressing the C-terminal construct were resolved by PAGE under non-reducing gel conditions, a higher molecular weight band was observed in cells treated with copper and cadmium, but not zinc-treated cells (Figure 5B). The band disappeared upon treatment of the cell extract with the reducing agent TCEP, but not upon incubation with the copper chelator BCS. This indicates that dimerization is reinforced by disulfide bond formation rather than by direct binding of the metal. To test if this covalent link is beneficial or detrimental for MTF-1 function, the zinc-induced activity of endogenous MTF-1 was measured on a 4xMREd-reporter in U2OS cells that were pretreated with different concentrations of copper for 16 hrs. As seen in Figure 5C, prolonged exposure to copper, which on its own is a poor inducer of MTF-1 in this mammalian system, clearly enhances MTF-1 driven reporter activity following zinc treatment.

3.5 MTF-1 activity can be partially rescued by a heterologous dimerization domain. In a one-hybrid experiment, for which the C-terminal part of MTF-1 covering all transactivation domains and the cysteine cluster (aa322-end) was fused to the Gal4 DNA-binding domain, the respective cysteine mutant reached, depending on the number of Gal4 binding sites in the promoter region of the reporter plasmid, upon zinc and cadmium induction up to 75 % and 50 % of the corresponding wild type protein activity, respectively (Figure 6). We consider it likely that the ability of the Gal4 DNA-binding domain to form homodimers [35] can

compensate for the loss of dimerization if the cysteine cluster is mutated. We can exclude that this rescue effect is merely based on the fact that eight Gal4-fusion proteins can bind to the promoter region of the reporter plasmid, since the activity of the cysteine mutant remains low if an analogous promoter with eight tandem MREs was used (Figure 6).

3.6 The cysteine mutant can still bind to p300. It was previously found that p300 interacts with mouse MTF-1 and that this interaction is enhanced by treating the cells with zinc [36]. The protein p300 is a ubiquitous coactivator protein of higher eukaryotes with histone acetyltransferase activity. We therefore tested if the cysteine mutant of human MTF-1 fails to activate transcription because its interaction with p300 was impaired. To this end, we transiently expressed tagged versions of p300 together with either wild type MTF-1 or the cysteine mutant in HEK293 cells. However, MTF-1 and p300 co-immunoprecipitated, which means that this interaction was independent of the presence or absence of the cysteine cluster (Figure 7).

4 DISCUSSION

The members of several families of eukaryotic transcription factors, like the bHLH, bZIP and the NF-κB transcription factors, need to dimerize to bind to regulatory sequences in their target genes [37]. Depending on the status of the cell, homodimers or heterodimers, typically within the same family of transcription factors, are formed that will activate a specific set of genes. Dimerization is not considered a common feature of C2H2 zinc finger transcription factors. In contrast to other DNA-binding domains the diversity of DNA binding specificities of C2H2 zinc fingers can be achieved by duplication and/or modifications of C2H2 motifs.

Dimerization was nevertheless reported for a number of zinc finger transcription factors. The transcriptional regulator Ikaros, which contains four N-terminal zinc fingers that mediate DNA binding, is capable of homo- and heterodimerizing with other Ikaros family members via another two zinc fingers located near the C-terminus [38-40]. Furthermore, a subfamily of C2H2 zinc finger proteins contains a highly conserved 84-residue motif, called the SCAN domain, that mediates self-association, as well as selective association with other proteins [41,42].

Here we show that human MTF-1 homodimerizes via a novel protein interaction domain, characterized by a cluster of four cysteines located near the C-terminus of the protein. This cluster is conserved throughout the vertebrate homologs of MTF-1, which underlines its functional importance. We show that transcriptional activity, whether tested on metallothionein or synthetic MRE-containing promoters, is dependent on the ability of MTF-1 to dimerize. Cell free binding studies indicate that, like the wild type protein, the cysteine mutant of MTF-1 can readily bind to DNA as a monomer. Using a one-hybrid approach we found that the effect of the cysteine mutant can be largely rescued by addition of the homodimerizing Gal4 DNA-binding domain. That the cysteine cluster can be functionally replaced by a heterologous domain mediating homodimerization suggests that it only serves this function and that neighboring domains, such as the acidic activation domain, need to be very close to each other to fulfill their function. However, the proximity between monomeric MTF-1's brought about by an array of MREs, like in the 8xMREd-OVEC reporter plasmid in which the MRE-motifs are separated by an 11 bp spacer, is not sufficient to rescue the cysteine mutant.

Even though binding of a metal ion to the cysteine cluster seems likely, zinc treatment did not increase MTF-1's dimerization as judged by the mammalian two-hybrid assay and co-

immunprecipitation results. Additionally, the interaction could not be destroyed by adding chelators for divalent metals. These data, together with the fact that also basal transcriptional activity is reduced in the cysteine mutant of MTF-1, suggest that induced dimerization is not involved in the sensing of elevated cellular zinc concentrations by MTF-1. Accordingly, the zinc-sensing mechanism is likely restricted to the zinc fingers and the acidic activation domain, as demonstrated previously [10,11,15]. However, we do not exclude binding of a metal ion to the cysteine cluster, independently of dimerization.

In the mammalian cell lines used, MTF-1 is poorly responsive to elevated concentrations of copper. Therefore it was a surprising finding that copper, as well as cadmium, enhance dimerization measured by a two-hybrid assay. Non-reducing gel electrophoresis showed that copper is able to oxidize the cysteines of the cluster so that a covalently linked dimer is formed. This effect was most readily observed after a prolonged treatment, perhaps indicating an exhaustion of cellular antioxidant protection.

So far it is not clear how the dimeric DNA binding of MTF-1 occurs. It is noteworthy that no conserved spacing between directly or divergently orientated MREs has been found in the promoter and enhancer regions of MTF-1 target genes. The possibility that dimerization is primarily required to bridge proximal and distant regulatory DNA sequences via looping out of the intervening DNA seems unlikely, since the cysteine cluster is also functionally important in a synthetic promoter with closely spaced, proximally located MREs. Furthermore, changing the orientation of MRE motifs in a synthetic promoter has no effect on reporter gene expression (U. Lindert and W.S., unpublished).

Taken together it may well be that only one partner of dimeric MTF-1 binds to DNA (see model in Figure 3C) and that dimerized MTF-1 is serving as a platform for the recruitment of factors of the transcriptional machinery, such as specific coactivator/Mediator components.

One candidate was the transcriptional coactivator p300 because on the one hand it was found to interact directly with mouse MTF-1 [36] and on the other hand human MTF-1 is acetylated by p300 *in vitro* (V.G. and W.S., unpublished). However, we show here that the cysteine mutant can still bind to p300. In *Drosophila* it was shown that several components of the TFIID and Mediator coactivator complexes are recruited to the metallothionein A promoter in response to copper [43]. It remains to be seen whether the mammalian homologs of these proteins are involved in a dimerization-dependent interaction with MTF-1.

Drosophila and mammalian MTF-1 are highly similar in their zinc finger domain while the C-terminal part which contains the activation domains is less, if at all, conserved [44]. Interestingly, despite the lack of similarity in the C-terminal part, *Drosophila* MTF-1 also contains a cysteine-rich cluster, though with a different spacing of cysteines (^{547}CNCTNCKCDQTKSCHGGDC^{565}) that is conserved among all drosophilid species. This cluster was shown to be necessary for copper-induced metallothionein gene transcription, a prerequisite for protecting the animals against copper toxicity. A different function of *Drosophila* MTF-1, namely, transcription of a copper importer gene in response to copper deprivation is not affected by mutation of this cysteine cluster [45], thus it is not generally required for transcriptional activity. The *Drosophila* cysteine cluster was shown to bind copper and accordingly is expected to sense high copper levels in the cytoplasm. In line with this model, replacement of the mammalian cysteine cluster with the one of *Drosophila* did not result in a functional protein [20]. We propose that even though MTF-1 is required for heavy metal homeostasis in both mammals and *Drosophila*, the respective cysteine clusters evolved independently and serve different functions.

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FOOTNOTES

The abbreviations used are: EDC, 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; MRE, metal response element; MTF-1, metal-responsive transcription factor-1; TCEP, *tris*(2-carboxyethyl)phosphine**;** TPEN, Tetrakis(2-pyridylmethyl)ethylenediamine; VSV, vesicular stomatitis virus.

FIGURE LEGENDS

Figure 1: Location and sequence alignment of the cysteine cluster.

(A) Functional domains of human MTF-1, showing the six zinc fingers, the acidic, proline-rich and serine/threonine-rich activation domains, the nuclear localization signal (NLS) spanning zinc fingers 1 – 3, a basic stretch of amino acids nearby with auxiliary NLS function

(aNLS), and the nuclear export signal (NES), which is embedded in the acidic activation domain. The conserved cysteine cluster is located in the C-terminal part of the protein. (B) Sequence alignments of the MTF-1 cysteine cluster region of *Homo sapiens* (human), *Mus musculus* (mouse), *Bos taurus* (cow), *Gallus gallus* (chicken), *Anolis carolinensis* (lizard), *Xenopus laevis* (African clawed frog), *Takifugu rubripes* (Japanese pufferfish) and *Oncorhynchus mykiss* (rainbow trout). For the rainbow trout the cysteine cluster differs somewhat from the typical pattern, and for the zebrafish *Danio rerio* no splice variant containing the cysteine cluster was described so far, however the putative reading frame is open and a variant containing the cysteine cluster is suggested by splice site predictions (K. Steiner and W.S., unpublished).

Figure 2: MTF-1 homodimerizes via its C-terminal half.

HEK293T cells were transfected with 2 µg of different VSV- and FLAG-tagged MTF-1 expression plasmids, followed by co-immunoprecipitations with an anti-VSV antibody. Immunoblots were developed with either anti-FLAG or anti-VSV antibodies. The failure of the N-terminal fragments FLAG-1-322 or FLAG-1-515 to co-immunoprecipitate with 322-end-VSV constructs is shown in (A). (B) Full length MTF-1 can homodimerize. (C) Co-immunoprecipitation of FLAG-322-end and 322-end-VSV reveal that the C-terminal half of the protein is sufficient for dimerization. WB: Western blot; IP: immunoprecipitation; α: antibody.

Figure 3: Dimerization of MTF-1 is dependent on the cysteine cluster.

(A) HEK293 cells were transfected with the indicated plasmids and whole cell lysates analyzed by co-immunoprecipitations with anti-VSV antibodies. (B) To determine the stoichiometry of the MTF-1 complex, whole cell extracts of HEK293T cells expressing MTF-1´s C-terminal part (322-end-VSV) were chemically crosslinked with EDC. Positions of molecular weight markers are indicated at the left of the figure. (C) Bandshift analysis using a ^{32}P-labeled MRE-s oligonucleotide and nuclear extracts of HEK293T cells that were

588 transfected with the indicated plasmids. On the right-hand side, the predicted DNA-protein
589 complexes that would explain the bands are shown schematically. (D) Co-
590 immunoprecipitations in the presence of 1,10-orthophenanthroline (Phen; 0.5 or 1 mM) or
591 N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; 0.1 or 0.2 mM) were performed
592 with whole cell extracts of HEK293 cells transfected with an expression vector containing
593 322-end-VSV revealing that the interaction persists in the presence of these metal chelators.
594 The chelators were added at the time of cell lysis, as they induce a rapid detachment of the
595 cells followed by apoptosis when added to live cells. WB: Western blot; IP:
596 immunoprecipitation; α: antibody.
597
598 Figure 4: MTF-1 dimerization is corroborated by a mammalian two-hybrid assay.
599 Two-hybrid interactions were measured in two human cell lines. HeLa or U2OS cells
600 were transfected with the 5xGOVEC reporter plasmid, Gal4- and VP16-fusion constructs and
601 OVEC-Ref or CMV-OVEC-Ref, respectively. RNA was isolated and expression levels of
602 reporter and reference genes were analyzed by the S1-nuclease protection assay. Reporter
603 gene transcript levels were normalized to reference gene transcript levels and compared to
604 the sample value of the “bait/prey” pair Gal-515-end-VSV/515-end-VP16, which was set to 1.
605
606 Figure 5: Copper synergizes with zinc induction of MTF-1 transcriptional activity.
607 (A) To test if metals have an effect on MTF-1 dimerization using the two-hybrid assay,
608 U2OS cells were transfected with 5xGOVEC, CMV-OVEC-Ref and Gal-515-end-VSV and
609 515-end-VP16 expression plasmids. Prior to harvesting the cells they were treated with
610 $ZnSO_4$, $CdCl_2$, $CuSO_4$ or menadione for the indicated times. Normalized values were
611 compared to the sample value of non-treated cells (n.t.), which was set to 1. The activity of
612 the “bait” construct Gal-515-end-VSV alone is marked by an asterisk. Error bars indicate
613 standard deviations of three independent experiments. (B) HEK293T cells were transfected
614 with 322-end-VSV expression plasmid and treated with 100 µM zinc, 400 µM copper or 30
615 µM cadmium for 16 hrs. Cleared cell lysates were separated by SDS-PAGE under non-

reducing and reducing (+TCEP) conditions and Western blots were developed with an anti-VSV antibody. Copper and cadmium, but not zinc, induces intermolecular disulfide bond formation. The copper chelator BCS was added to test if the upper band was eliminated by removing copper. (C) To analyze if copper pretreatment affects zinc-induced transcription via MTF-1, U2OS cells were transfected with 4xMREd-OVEC and CMV-OVEC-Ref and treated with different $CuSO_4$ concentrations 12 hrs before $ZnSO_4$ addition. The normalized value of untreated cells was set to 1.

Figure 6: Effect of the cysteine mutant is largely rescued by dimerization via a heterologous protein domain.

Dko7 cells were transfected with the corresponding reporter construct, OVEC-Ref and a MTF-1 expression plasmid or a MTF-1 fusion clone containing aa1-93 of the yeast factor Gal4, which has besides DNA-binding, also a dimerization function. Transcripts were measured by the S1-nuclease protection assay. Reporter signals were normalized to the reference signal and compared to the sample value of uninduced cells transfected with full length MTF-1, which was set to 1. Error bars indicate standard deviations of three independent experiments.

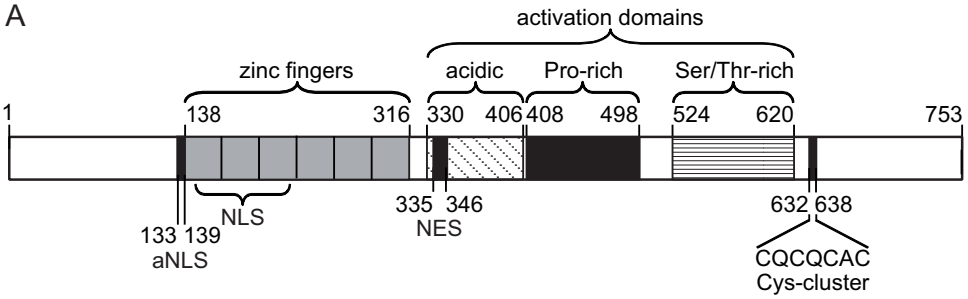
Figure 7: The cysteine mutant of MTF-1 can still interact with the transcriptional coactivator p300.

HEK293 cells that transiently express MTF-1-VSV or CYSmut-VSV together with FLAG-p300 were treated for four hrs with 100 µM $ZnSO_4$ where indicated. Whole-cell extracts were immunoprecipitated (IP) using an anti-FLAG antibody. An interaction, that is however independent of zinc supplementation, is revealed by immunoprecipitates which were analyzed by Western blotting (WB), using an antibody against the VSV- or FLAG-epitope.

Figure1

Figure 1

A



B

Cys-cluster

```
human          SSVQQIGLSVPVIIIKQEEACQCQCACRDSAKERASSRRKGCSSPP
mouse          SSVQQIGLSVPVIIIKQEEACQCQCACRDSAKERAAGRRKGCSSPP
cow            SAVQQIGLSVPVIIIKQEEACQCQCACRDSAKERAASRRKGCPSPP
chicken        NPVQQIGLSVPVIIIKQEEACQCQCACRDSAKDKVTVKKESSSPEP
lizard         SSVQQIGLSLPVIIIKQEEACQCQCACRDSAKEKAAAASAASSTSD
frog           NPVQQIGLSLPVLIIKQEESCQCQCACRDSAKDTAN-KRKGCSPAP
pufferfish     NSVQQIGLSLPVIIIKQEESCQCQCACRDSAKEKNSKSSSSSMSAQ
trout          TALQQLGLRLPVIVIRQGESCQCRCPCRDGSTASDTEKQTGCQPTG
               ..:**:** :**::*:* *:***:*.***.:.         . .
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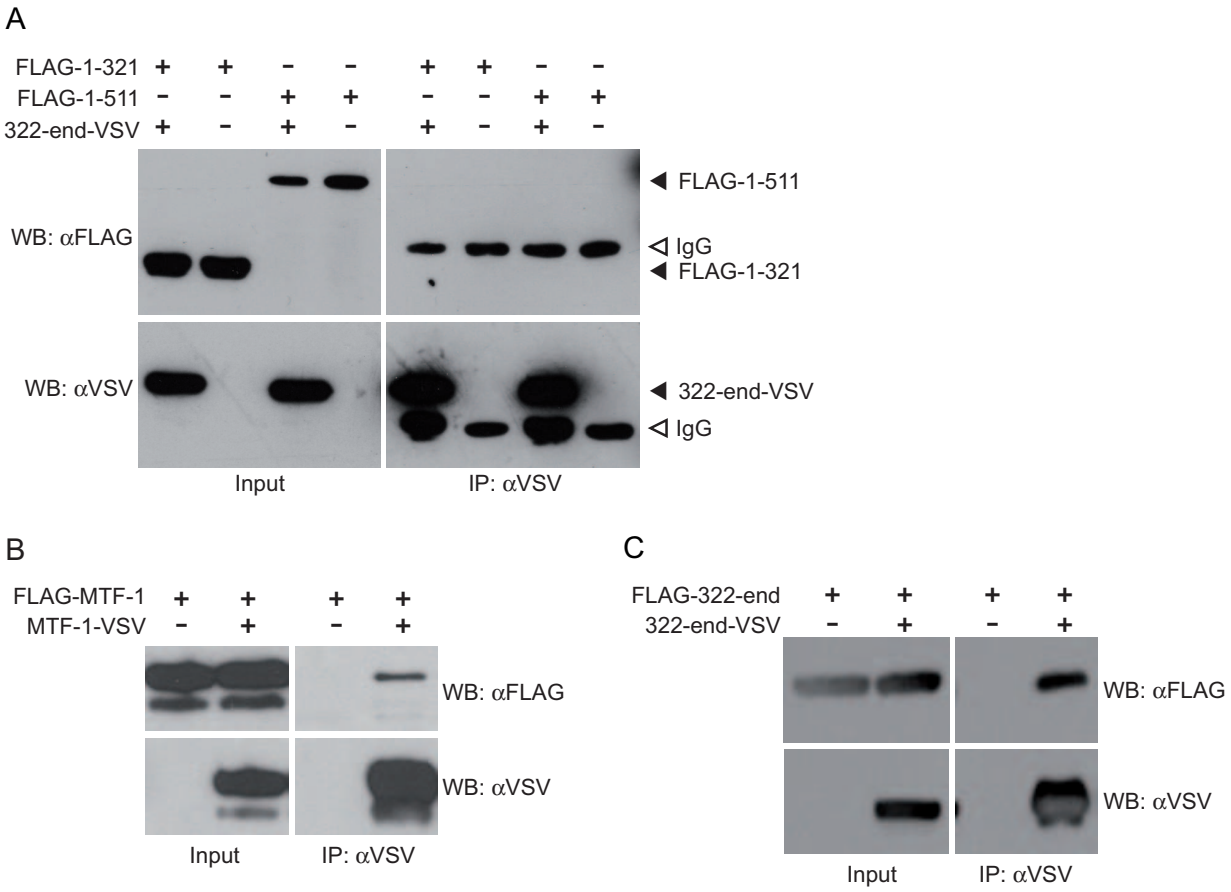
Figure 2


A
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FLAG-1-511 - - + + - - + +
322-end-VSV + - + - + - + -
WB: αFLAG
WB: αVSV
FLAG-1-511
IgG
FLAG-1-321
322-end-VSV
IgG
Input
IP: αVSV
B
FLAG-MTF-1 + + + +
MTF-1-VSV - + - +
WB: αFLAG
WB: αVSV
Input
IP: αVSV
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Input
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Figure3

Figure 3

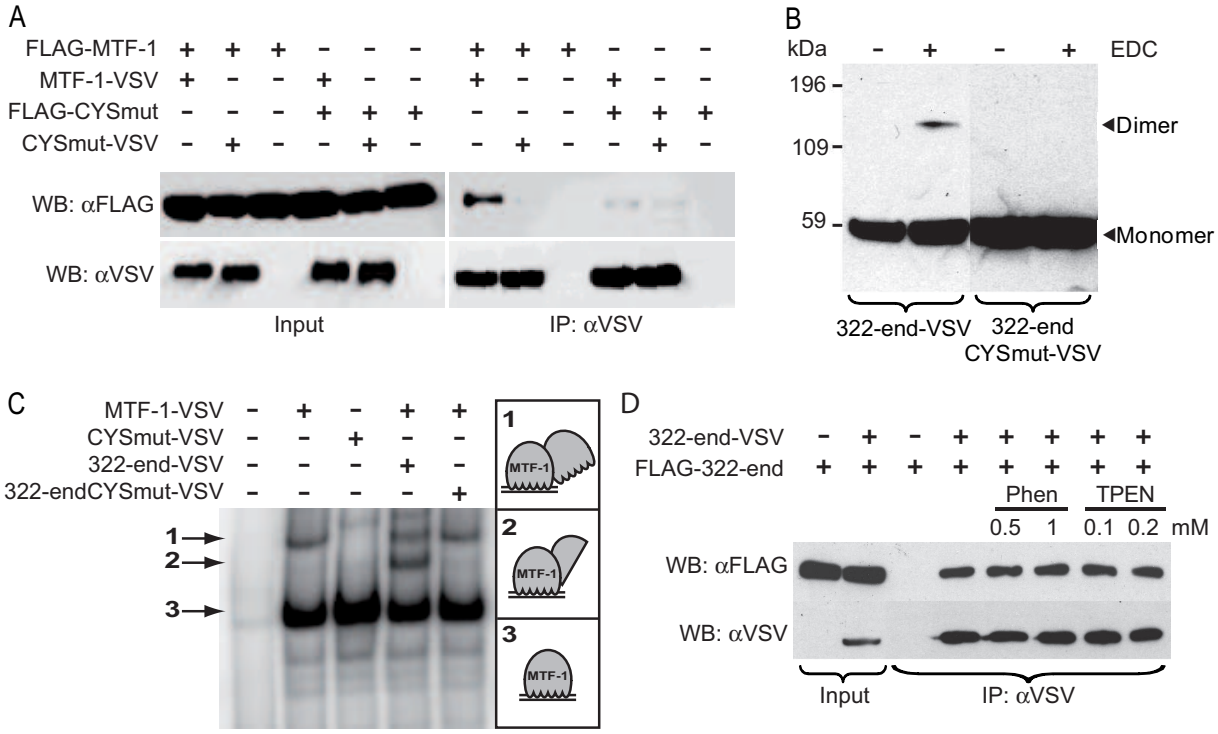


Figure4

Figure 4

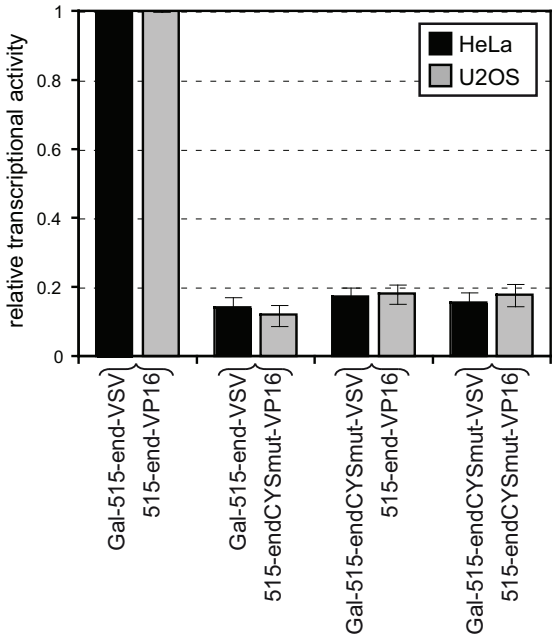
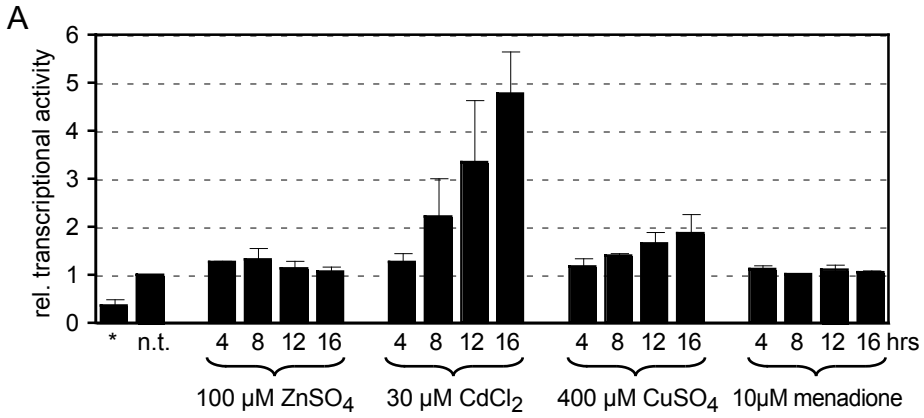
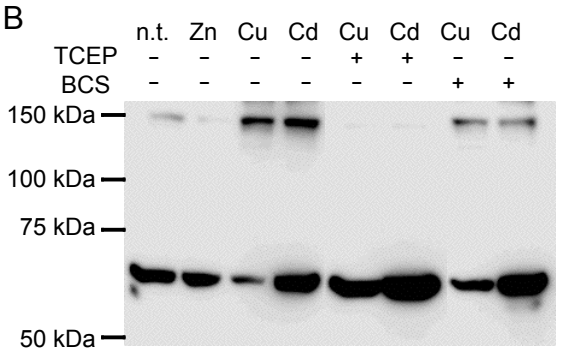


Figure5

Figure 5





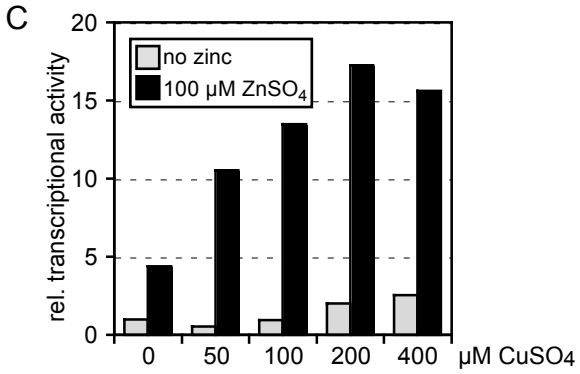


Figure6

Figure 6

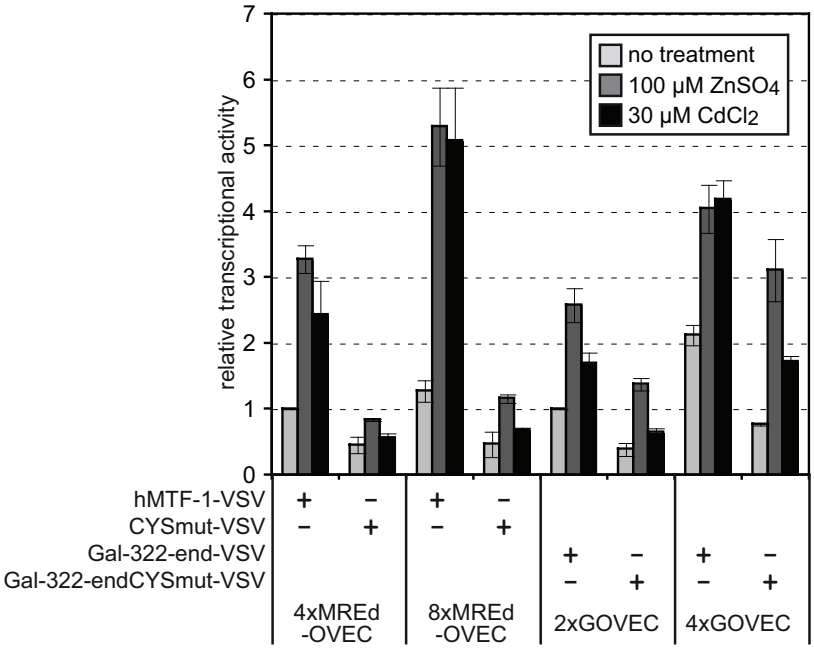
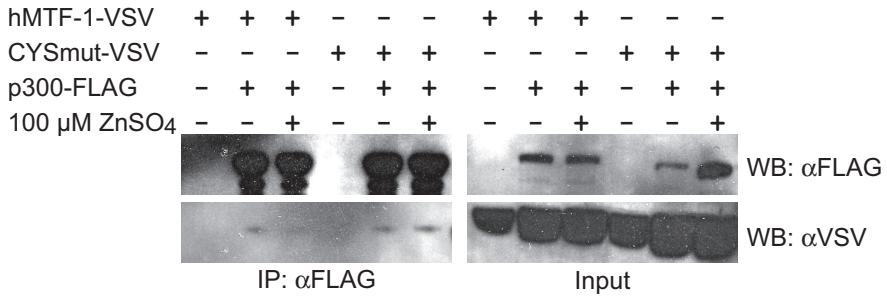


Figure7

Figure 7



Supplementary information

Materials and Methods:

Indirect immunofluorescence. Exponentially growing cells of a 10 cm dish were transfected with 1 or 5 µg (for HEK293T or U2OS cells, respectively) of the indicated expression plasmid and re-seeded on glass coverslips the next day. Cells were either left untreated, or treated with 100 µM $ZnSO_4$ for 3 hours. The immunofluorescence staining was performed as described previously [1] using a mouse anti-VSV (Sigma, V5507) and an Alexa Fluor 546 goat anti-mouse antibody (Invitrogen, A11030). Nuclei were stained by 4′,6-Diamidino-2-phenylindol (DAPI) at a concentration of 1 µg/ml for 1 minute.

Figures legends

Supplementary Figure 1: The cysteine mutant fails to activate transcription from MRE-containing promoters. Mouse cells lacking MTF-1 (dko7 cells) were transfected with the 4xMREd-OVEC reporter (in A), mMT1-OVEC [2] or hMT2a-OVEC [3] reporter (in B), OVEC-ref and 2 µg of the indicated wild type or mutant MTF-1 expression vectors. Cells were treated with the indicated metals for four hrs. Transcript levels were determined by the S1-nuclease protection assay and reporter signals were normalized to reference signals. The signal of cells transfected with hMTF-1-VSV treated with zinc, was set to 1. Error bars in indicate the standard deviation of three independent experiments.

Supplementary Figure 2: Nuclear localization upon zinc treatment and DNA binding are not disturbed by mutation of the cysteine cluster. The subcellular localization of hMTF-1-VSV or CYSmut-VSV was analyzed by indirect immunofluorescence in (A) U2OS and (B) HEK293T cells that were either left untreated (n.t.) or treated with 100 µM $ZnSO_4$ for three hrs. (A) For each condition 200 cells were counted and classified according to the following categories. N: nuclear localization only; C<N: mostly nuclear localization; C=N: equal distribution; C>N:

mostly cytoplasmic localization. (B) Immunofluorescent staining of transiently transfected HEK293T cells showing that the cysteine mutant retains the ability to translocate to the nucleus upon zinc treatment. (C) Electrophoretic mobility shift assay of a ^{32}P-labeled consensus MRE oligonucleotide (MRE-s, 40 fmol per reaction) with nuclear extracts (NUX) of dko7 cells that were not transfected (n.t.) or transiently transfected with either wild type or mutant MTF-1. Cells were treated with 100 μM $ZnSO_4$ for four hrs prior to cell harvest if indicated.

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Supplementary Figure 1

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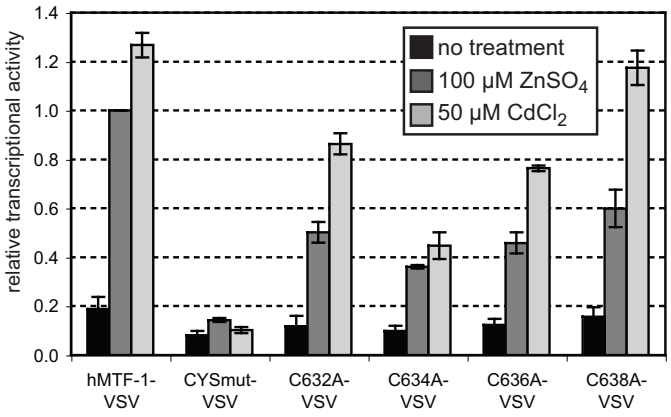
Supplementary Figure 1

A



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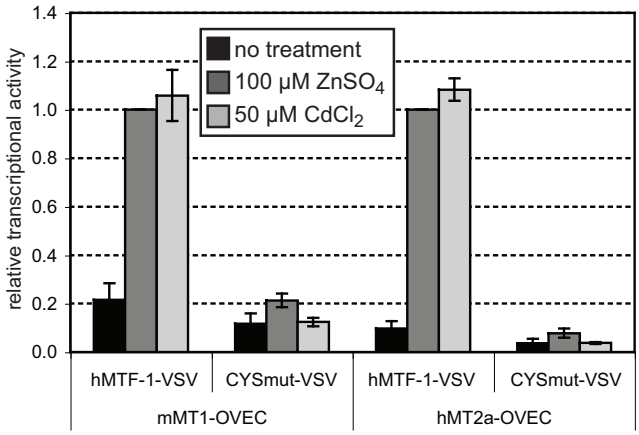

Supplementary Figure 2

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Supplementary Figure 2